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Xanthohumol in Brewing – Impact of Malt, Xanthohumol Dosage, Wort and Storage Temperature

The enrichment of the hop polyphenol xanthohumol (XN) in beer is limited because it is hardly soluble in water and it isomerises during wort boiling. Commercial dark beers comprise higher XN contents and in first investigations roasted substances proved to have potential for XN enrichment. In our brewing trials (10 and 50 L scale) we examined isomerisation in worts produced with different ratios of roasted malt beer. Moreover, we studied the point in time for XN addition to wort, as well as 23 malt and cereal varieties and a roasted malt liquid for the first time. In order to describe the XN carrier effect in more detail we compared gel permeation chromatograms of Pilsener, caramel, and roasted malt wort. Further, we conducted temperature stability experiments on XN in beer during pasteurisation and storage. The investigations confirm results from previous studies in which substances in dark roasted malts increase the total XN content in wort but still isomerisation takes place during wort boiling. New is the result that late addition of XN and rapid cooling of wort significantly reduce losses by isomerisation in dark brews. Moreover, the XN content increased with an increasing content of roasted substances in ceteris paribus wort. In tests on different malting products, we found an increasing XN enrichment potential with increasing EBC colour of roasted products. Intensively roasted malts contain predominantly high molecular weight (HMW) substances and are most effective for XN enrichment in the brewing process. Caramel malt wort with comparable EBC colour to roasted malt wort contained less XN carrying fractions with decreased XN contents. Stability tests showed that XN is widely stable during pasteurisation. Storage losses of XN increase with increasing storage temperature but decrease with increasing ratios of roasted substances in beer. We conclude that a range of roasting substances possess XN carrying and protective effects. Optimised roasting may improve this effect.

Descriptors: GPC, roasted malt, caramel malt, Isoxanthohumol, PVPP, roasted malt beer

1 Introduction

For more than 10 years, medicals and brewers have investigated the promising hop polyphenol xanthohumol (XN). XN has a broad medical spectrum of activities including antiviral as well as antioxidative effects [14,15,23]. Brewers see the opportunity to bring beer up for positive discussion because most people consume XN exclusively via beer. At first, the XN enrichment was investigated in pale brews. The prenylated flavonoid XN itself is a nonpolar polyphenol that is hardly soluble in water. In pale wort and beer, it is possibly bound to complex carbohydrates and other beer constituents like proteins. Equilibrium reactions between bound and unbound XN are discussed in this context [28]. Results, in which no maximum solubility of XN was found when increasing amounts of XN were added to wort corroborate this theory [33].

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Within the brewing process XN losses arise from isomerisation to isoxanthohumol (IX) during wort boiling. Further losses occur during precipitation, trub formation, fermentation, adsorption to yeast, storage, and filtration / stabilisation, so no more than 0.2 mg XN/L were found in commercial pale beers [10,12,28]. In a first attempt to reduce those losses, *Forster et al.* [11] added XN rich wort after the main fermentation ("feed") for the production of unfiltered beers [11]. Other technologies like the 'XAN-technology' include a high dosage of XN enriched hop extract at the end of wort boiling followed by a fast cooling of the wort to 80°C to reduce isomerisation of XN to IX. Further measures as the use of XN enriched yeast or dropping filtration and leave out stabilisation lead to XN contents between 1 and 3 mg/L in unfiltered beer [1,33].

Concerning dark brews, *Walker et al.* [30] and *Biendl et al.* [3] found XN contents up to 1.2 mg/L in Porter and Stout beers. Experiments with crystal malt, roasted barley and chocolate malt showed reduced XN losses due to inhibition of isomerisation during wort boiling. In pilot scale a filtered beer produced with these raw materials contained 3.3 mg XN/L [3,30].

Investigations on dark beer made from different malts revealed that roasted malts and roasted malt beer evolve especially high XN enrichment potential in beer. While at a first glance other selected malts revealed no correlation, roasted malts showed a linear corre-

lation between EBC colour and XN content in beer. A filtered beer produced with roasted malt contained 17 mg XN/L [33]. Further, these investigations showed higher XN contents in corresponding worts and reduced losses during fermentation, kieselguhr and sheet filtration. Treatment with polyvinylpolypyrrolidone (PVPP) resulted in a reduced XN content by 50 %, while in pale brews only traces of XN remained after this treatment. Gel permeation chromatography (GPC) of roasted malt beer revealed that especially high molecular weight (HMW) substances possess XN carrying effects. Most GPC fractions with high EBC colour were high in XN. However, uncoloured fractions contained few amounts of XN as well. At least to little extent, uncoloured substances may serve as XN carrier as well. Certainly a wide range of roasted substances carries XN through the brewing process [33].

Aim of this study is to point out the measures (a) malt variety and (b) point in time of XN dosage on the final XN content in beer in more detail. In doing so, we focus the binding between XN and HMW substances in worts made from different malts and its temperature stability in beer. The results should help brewers to increase XN contents in wort and beer as well as to avoid losses.

2 Materials and methods

Isomerisation experiments

In isomerisation experiments, 20 mg XN/L were pre-solved in ethanol (80 % XN, Xantho Flav-Pur®, Hopsteiner®, Mainburg, Germany) and were added to boiling wort under reflux conditions. Wort was boiled for 60 min after hop dosage of 50 mg α-acids/L (CO_2-extract). The colour was adjusted by addition of RMB into pale malt wort. The indication 0 EBC represents the pale wort without RMB. For darker worts an additional colour of 20, 40, and 80 EBC was given, respectively. Colour was measured according to ANALYTICA-EBC [9]. For comparison of brewing technologies we added 12 mg XN/L either to a brew according 'XAN-technology' [1], or to a conventional brew at the beginning of boiling. The latter wort was not precooled in the whirlpool. The fermentation took place at 12 °C for one week, followed by 2 days' maturation. After storage for 2 weeks, beers were filtered through sheets of KS200 (Pall Corporation®, Kreuznach, Germany).

Testing malt varieties and their XN enrichment potential

We investigated 21 commercial malts and 2 roasted cereals, which were all provided by Weyermann, Bamberg, Germany (Table 1). All malt and cereal samples were brewed in duplicate in 10 L quantities to study their XN enrichment potential in beer. Ratios of speciality malts within the grist load are listed in table 1. Hopping was done 5 minutes before end of boiling with an XN-enriched hop extract (2 % XN, Xantho-Flav®, Hopsteiner®, Mainburg, Germany) by an amount of 30 mg XN/L , according to 'XAN-technology' [1]. Then the wort was cooled down to 80 °C with cold brewing water. All brews were fermented at 12 °C for one week, followed by 2 days' maturation. After 2 weeks of storage, beers were filtered through sheets of KS200 (Pall Corporation®, Kreuznach, Germany). Additionally before dosage of the XN enriched hop extract, a Pilsener wort was adjusted to EBC wort colour comparable to a 5 % roasted malt 2 wort (80 EBC) by addition of roasted malt beer (RMB). The further processing was as described above.

Effect of variation of XN dosage and grist load of roasted malt on wort and beer

Worts with different grist loads of roasted malt 2 between 1 and 10 % were brewed in a pilot scale facility (50 L). After 60 minutes of boiling, the wort was separated into 5 parts, each 10 L. Then XN dosage in a range of 0 to 80 mg/L took place, followed by 20 min of whirlpool rest. Following process steps were as described above.

Impact of pasteurisation on the XN enriched beers

An unfiltered pale brew was separated into 5 parts. Different portions of XN enriched RMB were added to 3 parts until the desired colour (20, 40, and 80 EBC) was reached. The XN enrichment of the RMB was done with Xantho Flav-Pur® (80 % XN, Hopsteiner®) according to Back et al. [2]. Excluding one of the two pale brew parts without RMB, all beers were filtered. We tested the unfiltered and filtered pale beer without RMB and designated these as 0 EBC. After filtration, all beers were bottled and pasteurised. Two bottles of each beer were analysed after each run. The applied Pasteur units (PU) were ascertained with an ebro Pasteur-set and software winlog 2000 1.21 (ebro Electronic GmbH& Co. KG, Ingolstadt, Germany). A tunnel pasteurizer shield (Krones, Neutaubling, Germany) applied the PUs.

Impact of storage conditions on the XN content of enriched beers

As described for the brewing experiments for testing malt varieties, unfiltered pale worts were produced and separated into two parts. One part was enriched with 30 mg XN/L according 'XAN-technology'. To the other part an XN enriched RMB was added. Again XN enriched RMB was added before filtration to adjust EBC colour comparable to a 5 % roasted malt 2 brew. The stability tests were performed in separated compartments at 0 °C, 20 °C and 40 °C for 24 weeks.

PVPP treatment in beer

An amount of 500 mL lager beer was degassed at 12 °C and enriched with XN enriched RMB (produced according Back et al. [2]). Then the beer was stirred and PVPP (Sigma Aldrich, Germany) was added in an amount of 50 g/hL. After 10 minutes of stirring PVPP was separated by centrifugation. This procedure was repeated 6 times to identify strength of XN bonds.

Characterisation of XN carrier

In accordance with results on size exclusion chromatography presented by *Wunderlich et al.* [33], fractionated worts were prepared from Pilsener malt, caramel malt 5 and roasted malt 2 by gel permeation chromatography (Superdex 200 column, Amersham Bioscience). The XN content was determined in fractions with and without PVPP treatment, which was again applied to identify strength of XN bonds [33].

HPLC analyses

XN and IX was determined in duplicate in degassed and centrifuged wort and beer samples as described by our research group in 2005 [33]. A sample volume of 25 µL was directly injected on a high performance liquid chromatography – diode array detector (HPLC-DAD) system (Hewlett Packard 1090 Series II, column: Macherey-Nagel EC-250/4 Nucleosil 100-5 C18 hop). DAD wavelength was 375 nm for XN and 290 nm for IX detection. Quantification was done

Table 1 colour and grist load ratio of the investigated malts, cereals, and roasted malt liquid

product	trade name	colour [EBC]	grist load [%]
brew malt			
Pilsener malt (pale malt)		5	100
Munich malt (color/ dark malt)		40	100
special malt			
smoked malt		6	100
acidulated malt		7	5
melanoidin malt		80	20
chit malt		4,5	20
caramel malt			
caramel wheat malt	CARAWHEAT®	100	15
caramel malt 1	CARAHELL®	30	30
caramel malt 2	CARARED®	60	25
caramel malt 3	CARAAMBER®	80	20
caramel malt 4	CARAMUNICH® type II	130	10
caramel malt 5	CARAAROMA®	400	15
roasted malt			
roasted malt 1	CARAFA® type I	950	5
roasted malt 2	CARAFA® type II	1100	5
roasted malt 3	CARAFA® type III	1300	5
roasted malt 4 dehusked barley	CARAFA® SPECIAL type I	950	5
roasted malt 5 dehusked barley	CARAFA® SPECIAL type II	1100	5
roasted malt 6 dehusked barley	CARAFA® SPECIAL type III	1300	5
roasted rye malt		800	5
roasted spelt malt		600	5
roasted wheat malt		1050	5
roasted unmalted grains			
roasted barley		850	5
roasted rye		600	5
roasted malt liquid			
roasted malt beer (RMB)	SINAMAR®	8300	As necessary

applying an external XN and IX standard solution (Phytochem®, Ichenhausen, Germany).

3 Results and discussion

Isomerisation experiments

In wort, isomerisation of XN into IX depends on temperature. In aqueous, acidulated (HCl) solution [22] as well as in buffered sucrose solution [28] the isomerisation follows a first order kinetic. Lowering temperature from 100 °C to 80 °C led to significantly reduced isomerisation of XN in pale worts [33]. Roasted substances e.g. originated from roasted barley or RMB, inhibit the XN isomerisation [3,18,30,33]. Figure 1 displays the isomerisation of XN in wort produced with different ratios of RMB. The figure shows that the XN content in wort increases with increasing RMB ratio at equal XN dosage. This effect occurs throughout the boiling process. The relative loss comes to 70 % for all worts while the absolute losses increase from the beginning to the end of boiling.

We assume that the isomerisation from XN to IX influences the equilibrium reaction between unbound XN and XN that is bound to roasted substances. While XN isomerises, the content of unbound XN decreases in wort. This results in a shift within the equilibrium of bound and unbound XN so more XN is released from the carrier that can be isomerised. Figure 1 clearly shows that boiling releases XN from carrier substances. Previously we assumed a range of substances that act as carrier [31,33]. Possibly the strength of the binding between XN and these carrier varies so XN is bit by bit released during the ongoing boiling process. However, the equilibrium is balanced in favour of the roasted substances because in dark worts more XN is recovered at an equal XN dosage. When calculating the reaction kinetics [19] from results in pale and different dark worts, we could not specify a definite reaction order

for the decrease of the XN content. Both, first and second order reactions gave reasonable results. The coefficient of determination for the first order kinetic was between 0,985 and 0,997 and for the second order kinetic 0,975 and 0,997, respectively. We assume that different effects overlay each other. Beside the significant effect caused by roasted substances, reactions between XN and pale wort substances occur.

The isomerisation experiment illustrates the importance of late XN dosage for XN enrichment in dark beer containing roasted substances similar to effects in pale beer that we previously reported [33]. We would achieve threefold XN content in wort at the end of boiling when we applied this procedure compared to XN dosage at the beginning of boiling. The improvement in XN yield is obtained without additional colouring and at equal XN dosage.

We compared two brews produced with a XN enriched hop extract to investigate preservation of the increased XN content in dark wort throughout the brewing process. The XN product was added (1) at the beginning of boiling and (2) 5 min before the end of boiling followed by a fast cool down to 80°C ('XAN-technology', Fig. 2). In brew (1) the XN content increases after addition and decreases during boiling due to isomerisation. A low rise in the XN content during whirlpool rest may be explained by sample preparation. Particles like proteins reduce efficiency of centrifugation and filtration during sample preparation. The particle content is higher in boiling wort compared to pitching wort after the whirlpool, where particles are separated before sampling. Figure 2 shows that in brew (1) the XN content significantly decreases during boiling, fermentation, and filtration.

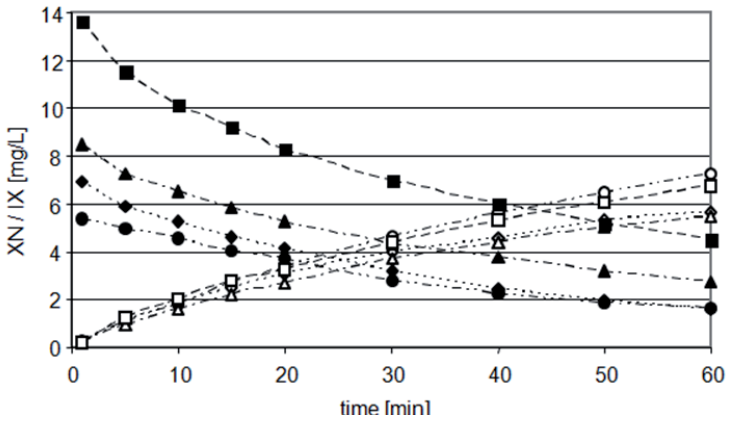

Fig. 1 Isomerisation of XN during wort boiling with different parts of RMB (– ■ – 80 EBC XN; – □ – 80 EBC IX; – ▲ - 40 EBC XN, – △ - 40 EBC IX; ·· ♦ ··20 EBC XN,·· ◊ ·· 20 EBC IX; – ● -- 0 EBC XN; – ○ -- 0 EBC IX)

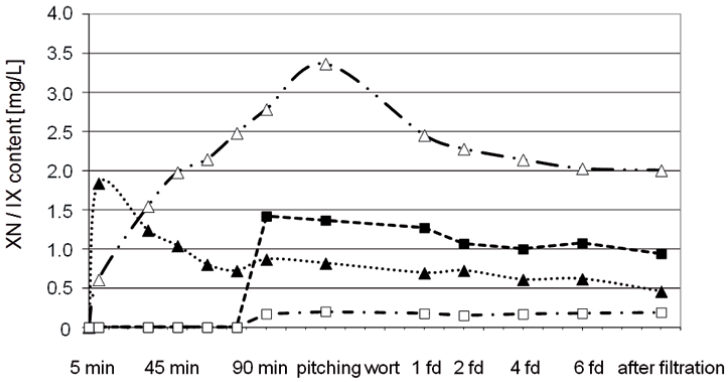

Fig. 2 XN and IX content during conventional brewing and brewing according the 'XAN' technology. (·· ▲ ·· XN conventional brew; ·· △ ··IX conventional brew; – ■ - XN XAN brew, – □ - IX XAN brew) [31]

The XN content after addition of XN in brew (2) (1.3 mg XN/L) does not fully level up with the maximum XN content in brew (1) (1,9 mg XN/L), although the same amount of XN was added (Fig. 2). This is ascribed to a reduced period that hinders complete solution of the XN. Late XN dosage and the precooling with cold brewing water in the whirlpool reduce XN losses within the brew house. The IX content remains constant on a low level, while it originates partly from isomerisation and partly from the hop product that also contains IX or both. As well as in brew (1) XN losses occur during fermentation and filtration in brew (2). However, the final XN content is around 0.5 mg/L in brew (1) and 1 mg/L in brew (2). This confirms the finding from above that the addition of XN at the end of boiling and precooling in the whirlpool is advantageous for production of XN enriched beers. As mentioned previously, "precooling of wort to 80 °C may lead to a deficient formation of the trub cone, resulting in an increased carryover of hot trub to the fermentation tank" [33]. On the other hand, the kieselguhr containing hop extract used for this study may facilitate the trub sedimentation. Concerning the XN content, we note that this procedure guarantees high XN yields in the final beer. The associations between XN and carrier substances are more intensively affected by boiling than by fermentation and filtration. Applying the described brewing technology, we were able to double the XN content in filtered beer compared to a brew produced according to conventional brewing technology.

Testing malt varieties on their XN enrichment potential

In this study we extended the range of malt varieties and roasted cereals compared to our study from 2005 [33] to investigate correlations between XN and roasted substances in more detail [33]. Figure 3 displays a positive correlation between XN yield and EBC colour in beer. The same trend emerges from XN yield versus EBC colour in wort (data not shown). Beers produced with dark speciality malts below 400 EBC malt colour reach XN contents up to 0.7 mg/L. These yields are independent from the corresponding grist loads, which ranged between 5 and 25 %. In all other brews the grist load was 5 %, so that increasing beer colour corresponds to intensity of malt roasting. Over all beers the calculated linear regression between XN content and EBC colour results in a coefficient of determination (R^2) = 0.77, the logarithmic model gives R^2 = 0.71. Both R^2 improve to 0.90 (linear) and 0.82 (logarithmic), respectively, when the caramel malt 5 brew is excluded. However, produced with a grist load of 15 % and a beer colour of 80 EBC, the caramel malt 5 brew reaches only 0.3 mg XN/L. A brew produced with roasted malt 2 (5 % grist load) contains 5 mg XN/L at the same EBC beer colour. Wunderlich et al. [33] observed 2005 the difference in XN contents in caramel and roasted malt wort and concluded that different Maillard products are responsible [33]. Studies by *Coghe et al.* [6] and Forster et al. [13] reveal that similar EBC beer colours may arise from different colouring substances in speciality malts [6,13]. E.g. the Pilsener malt colour originates from low molecular weight (LMW) colouring substances < 10 kDa, while predominantly HMW substances >

100 kDa are responsible for colour in roasted malt [7,8]. Both malts are produced in a roasting drum. Caramel malt is made from green malt, a rest between 40 °C and 50 °C is usual but the saccharification rest by 60–75 °C is obligate. In general, maximum roasting temperatures lie between 120 and 180 °C [4,21]. Roasted malt is usually made from kilned pale malt. It is often moistured at 70–80 °C and a kind of saccharification rest is held before roasting. The end temperature is in the range of 220 and 240 °C. In addition, roasting takes longer for roasted malts than for caramel malts [4,21]. Coghe describes a correlation between development of different colouring substances and the temperature at the end of roasting [7]. In contrary for the tested malts caramel malt 5 and roasted malt 2 Kraus-Weyermann reports just small differences between the final temperatures (caramel malt: 180– 210 °C; roasted malt: 180–220 °C) [20]. We assume that not only the final temperature is important for formation of XN carrier but the preroasting treatment as well (e.g. counts and intensity of rests). Model studies have shown that the development of key intermediate products of the Maillard reaction depend on the moisture content as well [5,16]. Maybe caramel malt contains less XN carrier due to consumption of precursor for e.g. flavour compounds. This may result in less accumulation of XN carriers and/or the duration of roasting was not excessive enough.

The ratio of colour:XN is a suitable parameter to specify colouring per 1 mg XN. This is especially relevant when pale XN enriched beers are aimed and the proper malt has to be chosen. This ratio decreases with increasing roasting intensity (Fig. 4). Less colouring occurs per 1 mg XN when more intensively roasted malt is used. E.g., the ratios were 44 EBC/mg XN for roasted rye and 16 EBC/mg XN for roasted malt 2 in our trials (Fig. 4). Exceptions are very pale malts like Pilsener malt that has a low EBC colour but a certain XN enrichment potential. This is possibly due to mechanisms like association to proteins or polysaccharides. As consequence the calculated colour:XN ratio is low but the malt is not suitable for XN enrichment in practice. In contrast, the RMB brews resulted in a ratio of 12 EBC/mg XN. We guess that RMB is made from particularly intensively roasted malt, which is especially rich in XN carrying substances. We developed a special XN enriched roasted malt extract with an even lower colour:XN ratio, based on these results [2]. The XN enriched roasted malt extract represents a customized solution for industrial production of an XN enriched beer and can e.g. dosed before or after filtration.

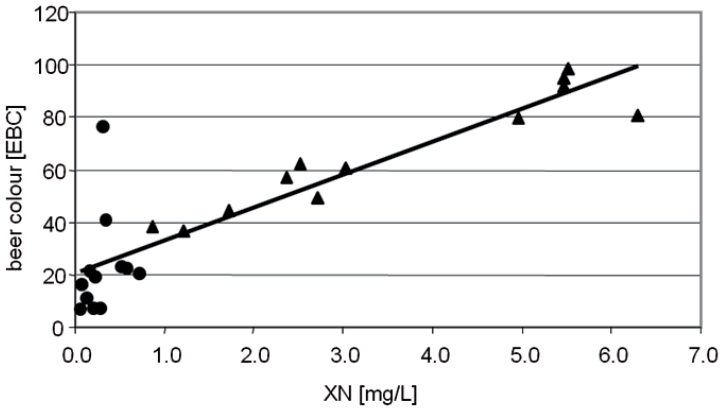

Fig. 3 Beer colour and XN content of beer (● made without roasted products; ▲ made from roasted products with 5 % grist load) [31]

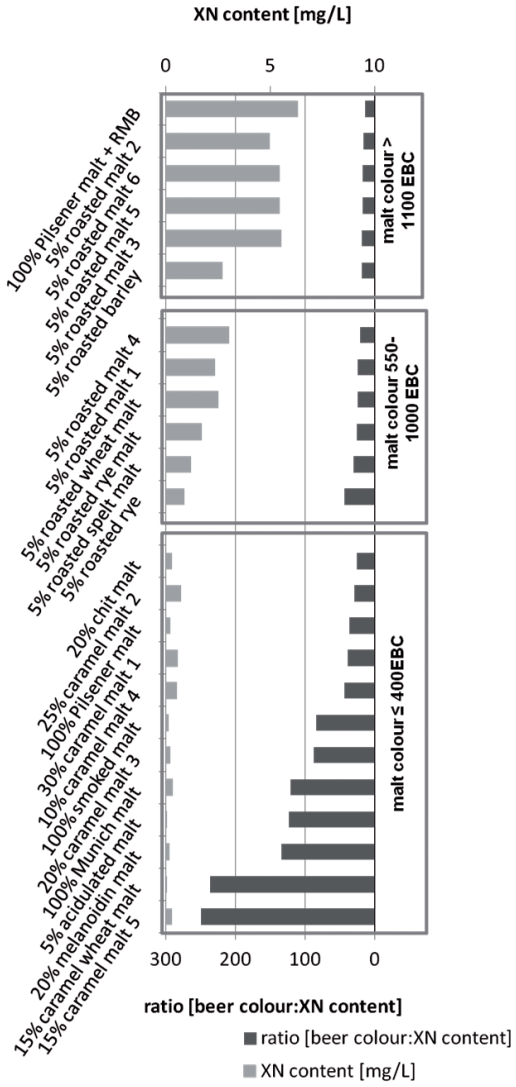

Fig. 4 Pilsner in Pilsener

The IX content increases with increasing roasting intensity as well (Fig. 5). However, the effect is much more pronounced for XN as indicated by a faster increase of the XN content compared to the increase of the IX content (slope < 1). In general, IX is more polar than XN. The resulting higher solubility in wort makes it less susceptible to separation processes like precipitation and filtration [28]. Probably IX is also carried by roasting substances as well as other substances similar to XN like e.g. 8-prenylnarigenin. The latter was found at increased amounts in Stout beers [26].

Impact of variation of XN dosage and grist load of roasted malt on wort and beer

In pilot scale brewing experiments, we varied the XN dosage and the grist load of roasted malt 2. The resulting beers cover a wide spectrum of combinations between XN content and EBC colour (Fig. 6). The extremes highlight the effects of each parameter. XN recovery increases with increasing roasting malt ratio and XN dosage. The effect of roasted malt 2 exceeds the effect of XN dosage. E.g. the increase in XN dosage from 40 to 80 mg/L at 10 %

roasted malt 2 ratio results in XN contents of 11.2 and 17.2 mg/L, respectively. Doubling the roasted malt 2 ratio from 5 to 10 % at an XN dosage of 80 mg/L causes XN contents of 8.2 and 17.2 mg/L, respectively. A correlation occurs between both, the roasted malt ratio as well as the XN dosage, and the final XN content in beer. Best results gave a linear model between roasted malt ratio and final XN content in beer ($R^2 > 0.98$) that confirms results obtained in a previous study [33]. The correlation between XN dosage and final XN content in beer is fitted by a logarithmic model ($R^2 > 0.98$).

In our experiments, the losses during fermentation, maturation and storage exceed losses during filtration. In both process sections the losses decrease with increasing roasted malt ratio. At an XN dosage of 40 mg/L, the brew with 1 % roasted malt 2 contains 5.8 mg XN/L in pitching wort, and 1.9 mg XN/L before filtration (Fig. 6). This equals to an absolute loss of 3.9 mg XN/L (approx. 10 % of initial dosage). We detected 0.9 mg XN/L in the resulting beer, which is equivalent to a relative loss of 2.3 % of the initial dosage of 40 mg XN /L during filtration. The brew with 10 % roasted malt 2 ratio at the same initial XN dosage reveals the effect of roasted malt on the absolute XN content in the brew. The pitching wort contains 17.5 mg XN/L. Before and after filtration 13.4 and 11.6 mg XN/L were measured, respectively. However, the relative losses within fermentation, maturation, storage, and filtration compare to the relative losses in the 1 % roasted malt 2 brew (10 % between pitching wort and before filtration, and 4.5 % during filtration) (Fig. 6).

XN yields depend on XN dosage and the roasted malt 2 ratio in the grist load, as well. They range between 1 (dosage: 80 mg XN/L, roasted malt: 1 %) and 40 % (dosage: 10 mg XN/L, roasted malt: 10 %). Moreover, we were able to reduce the colour:XN ratio (160 EBC:17.2 mg XN/L) to 9.3 EBC/mg XN in filtered beer (dosage: 80 mg XN/L, roasted malt: 10 %, Fig. 6) compared to previous studies [33].

Characterisation of XN carrier

Beers produced with Pilsener malt, caramel malt 5, or roasted malt indicate points of special interest regarding the XN enrichment potential in a wide range of malt varieties. Our studies, presented in 2007 [31], showed that especially caramel malt 5 takes a particular position concerning the XN carrier effect. This result was confirmed

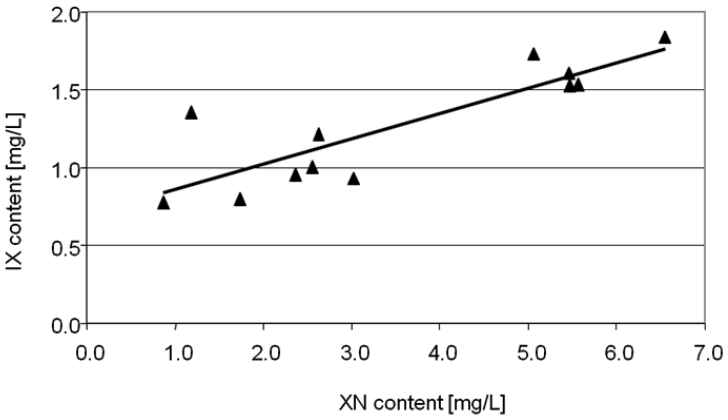

Fig. 5 IX against XN content in beer exclusively brewed with roasted malt or roasted cereals (▲ beer made from roasted products; $R^2 = 0.73$)

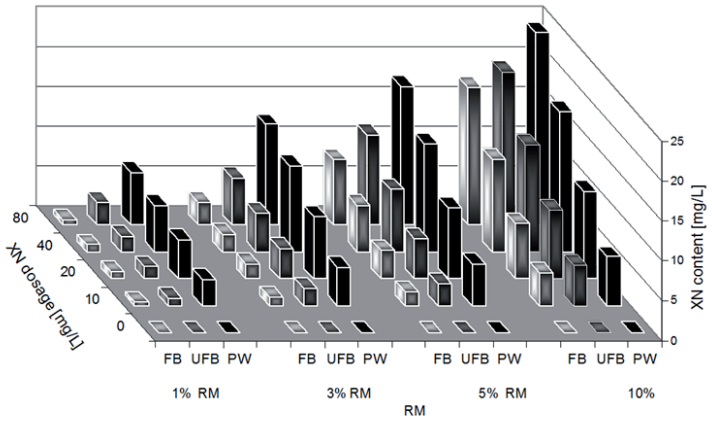

Fig. 6 XN content of pitching wort, unfiltered and filtered beer by variation of XN dosage and roasted malt ratio (PW: Pitching wort; UFB: unfiltered beer; FB: filtered beer, RM: roasted malt 2) [31]

by other authors in 2011 [18]. In 2007 [31] we already reported that although high in EBC beer colour (similar to roasted malt 2 brews), the caramel malt 5 brew exhibits XN contents in the range of Pilsener malt brews (Fig. 4). GPC analyses of wort should lead to a better characterisation of XN-retaining substances within the three malt varieties (Pilsener malt, caramel malt 5, roasted malt 2). The GPC differentiates wort constituents according to their molecule size. The molecular size decreases with increasing count number of the fractions. Figure 7 displays XN contents before and after PVPP treatment as well as the colour of all fractions. As we stated in 2007 [31], the results show that the XN content corresponds to wort colour in the different GPC fractions. PVPP treatment does not remove all XN in roasted malt worts. Compared to the roasted malt 2 wort, Pilsener and caramel malt 5 worts contain significantly less XN carrying fractions and also the sum of XN contents over all fractions is reduced. The Pilsener wort exhibits an especially low XN content. Therefore, we chose a smaller scale for visualisation. It is conceivable that not all colouring substances are responsible for XN enrichment.

In previous works, ultrafiltration in combination with GPC showed that XN carrying substances have a molecule size between 300.000 and 600.000 Da [31,33]. Further this filtration ensured that no secondary interaction between analyte and stationary phase occurred [17]. Our results comply with studies by Coghe [6] who stated that in Pilsener malt (malt colour < 150 EBC) 70% of the total colour is caused by LMW substances <10 kDa. Colour of roasted malt (malt colour > 800 EBC) predominantly arises from HMW substances > 100 kDa that reach a mean molecular size of 320 kDa. Caramel malt 5 contains LMW (similar to those in Pilsener malt) and HMW (similar to those in roasted malt) colouring substances in almost equal proportions. GPC analyses of malt samples taken during the roasting process showed a decrease in LMW and an increase in HMW substances. Resulting chromatograms described by Coghe [6] are comparable to our GPC results obtained from conventional malts (Pilsener malt, caramel malt, roasted malt). As previously shown, intensive roasting produces XN carrier that are HMW substance between 300 and 600 kDa [6,7].

After PVPP treatment differences in XN recovery within the GPC fractions 1–11 from caramel malt 5 and roasted malt occur. This observation in combination with higher XN-contents and more XN containing fractions from roasted malt suggest that it is a matter

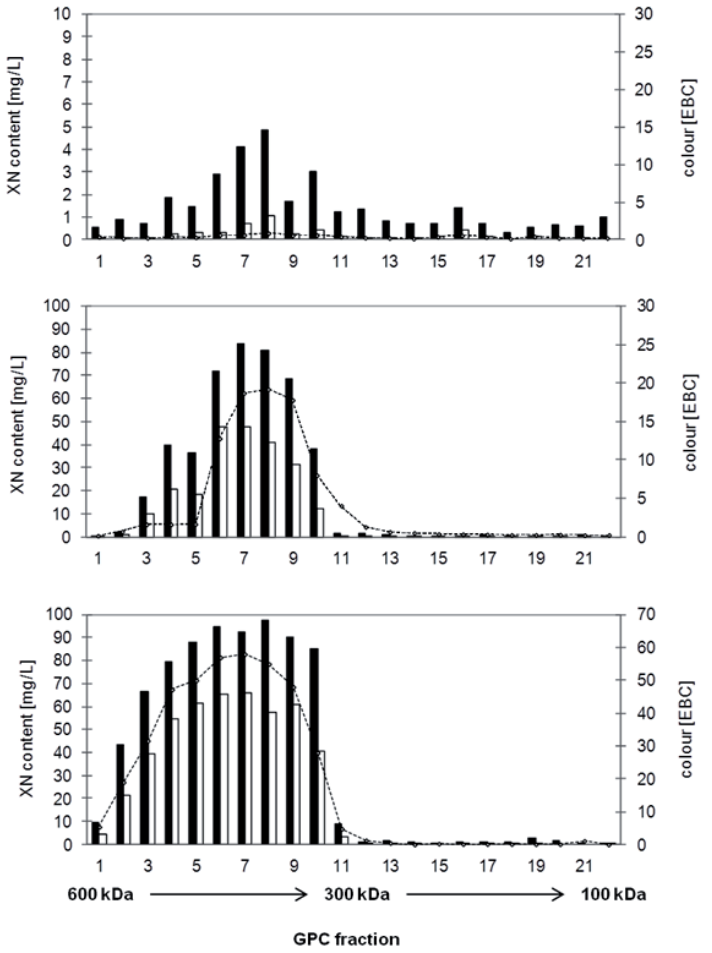

Fig. 7 XN content before and after PVPP treatment of XN enriched GPC wort fractions [31]

of not only one but a variety of substances. These substances are characterised by a high affinity to XN. We assume that smaller molecules that are not separated by ultrafiltration and GPC (< 100 kDa) also associate XN. But, in contrast, there a reduced strength of binding may ease the release of XN and sorption to PVPP [31,33].

Impact of pasteurisation on XN enriched beers

For the producer of beverages the stability is particularly important from the beginning of production until the end of the shelf life of the final product. Pasteurisation is still used to make beer more stable against beer spoilers. Forster et al. [10] ascertained a relative loss in the range of 3 to 5 % in pale brews when applying 30–40 Pasteur units. We produced an unfiltered pale malt beer and added increasing amounts of a XN enriched RMB before filtration. The brew without XN enriched RMB (no colour added, 0 EBC beer in Fig. 8) had the lowest XN content. It is the same brew than the 0 EBC unfiltr. beer (Fig. 8) but filtered. The losses up to 44 Pasteur units are negligible with up to 6 % loss in all brews (Fig. 8). At the end of the trial (620 PU) the losses were between 12 % (80 EBC) and 30 % (0 EBC unfiltr.). The losses decreased with increasing colour. The losses in XN were quantitatively found as IX in the beer. Keeping in mind that conventionally less than 40 Pasteur units are applied for pasteurisation of lager beer, we conclude that conventional pasteurisation has a negligible effect on the XN content.

Effect of storage conditions on the XN content of XN enriched beers

In order to study the thermal effect on the XN stability in beer, we produced an unfiltered pale brew with 1.10 mg XN/L and a dark beer made with XN enriched RMB (8.60 mg XN/L). The unfiltered pale beer showed losses at 40 °C within two weeks (Fig. 9). After 24 weeks storage at 40 °C 12 % of the initial XN concentration was retained. Storage at 0 °C and 20 °C showed first losses after 8 weeks. At the end of storage 90 and 55 % XN were retained in the pale 0 °C and 20 °C brew, respectively.

In the dark filtered brew storage led to losses as well, but after 24 weeks 22 % XN remained in the 40 °C brew (Fig. 10). Compared to the unfiltered pale brew the XN carrier supports the stability of XN during storage. However, in pale and dark brews the XN losses were not totally recovered in IX contents like in the pasteurisation experiments. We assume that XN and especially IX further degrade to unknown breakdown products. In laboratory scale experiments, *Stevens et al.* [28] showed that no side reactions occur when XN

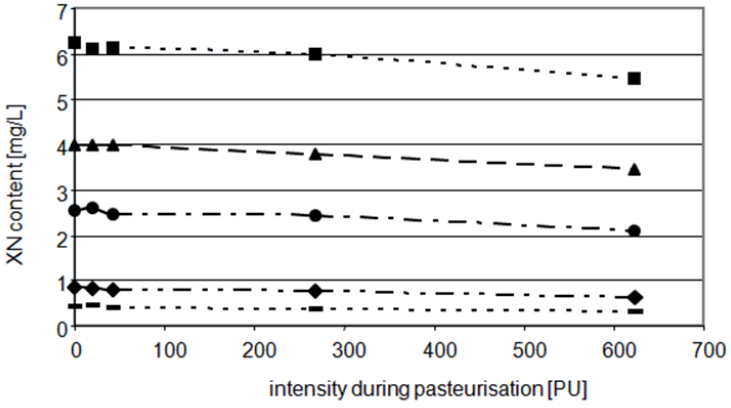

Fig. 8 XN content of beer made with different portion of a XN enriched RMB during pasteurisation. (-- ■ -- 80 EBC; – ▲ – 40 EBC, – ● - 20 EBC; – ♦ -- 0 EBC unfiltr. beer; – -- 0 EBC beer) [31]

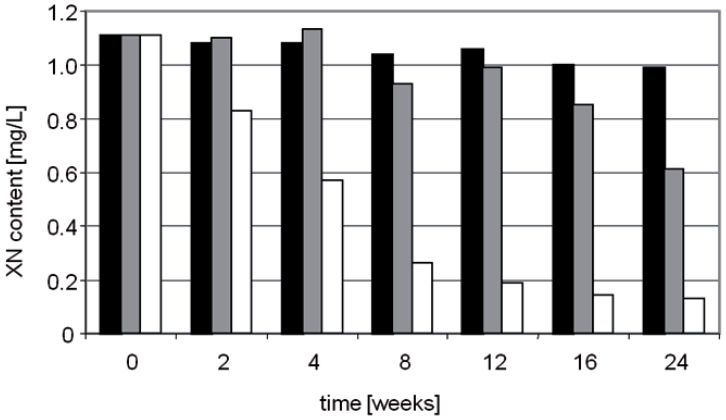

Fig. 9 Storage of unfiltered pale beer by different temperatures (■ 0°C; ■ 20°C; □ 40°C) [31]

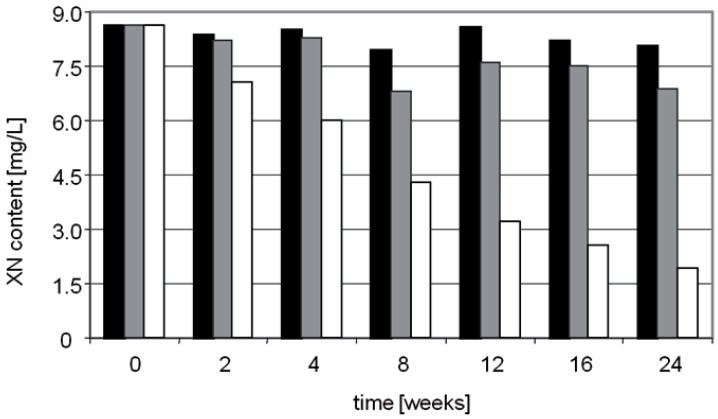

Fig. 10 Storage of dark beer made with XN enriched roasted malt beer by different temperatures (■ 0°C; ■ 20°C; □ 40°C) [31]

is isomerised into IX in a buffered sucrose solution. Similar to our results the authors couldn't recover the total XN losses in wort by IX [28]. During fermentation, the XN losses were caused by interaction with yeast. Possibly XN adsorbs to yeast surface because it is more lipophilic than other polyphenols like anthocyanoidines or phenolic acids. Therefore XN develops a stronger affinity to lipophilic surfaces like membranes and low density proteins [27]. Another reason for XN losses may be that yeast metabolises the compound [24,33]. Excluding such losses, XN seems to be relatively stable against chemical or microbial degradation by other microorganisms than yeast. However it is reported that the intestinal bacterium *Eubacterium limosum* metabolises IX to 8-prenylnarigenin by o-demethylisation [25]. More investigations are in demand on adsorption and IX degradation to understand these losses fully.

We conclude from the storage experiments that storage at low temperatures is advantageous for beer quality and XN stability, especially regarding that the storage conditions we used were more intensive then conditions used for flavour stability tests in general.

PVPP treatment in beer

We treated XN enriched beer several times with PVPP to investigate the binding between XN and XN carrier substances. Starting at an initial XN content of 2.05 mg XN/L the measured values logarithmically decrease with increasing amount of treatments (R^2 = 0.996, Fig. 11). We assume equilibrium reactions cause this effect, similar to our observation in the isomerisation experiment. Repeatedly a certain XN ratio is released and extracted by PVPP. We obtained similar results during investigations on efficiency of solid phase extraction (SPE) as sample cleanup in the XN analysis. Although different binding mechanisms occur during SPE (C 18 sorbent, nonpolar interaction e.g. van der Waals forces) [29] compared to PVPP treatment (polar interaction, hydrogen bridge bonds), repeated SPE showed decreasing XN recoveries [32]. In combination with results from repeated PVPP treatment, we conclude that the binding between XN and roasting substances differs in its type and strength. However, this binding can be released by HPLC conditions (acetonitril-water mixture). More experiments are required to describe the bindings between XN and roasting substances as well as their properties in further detail.

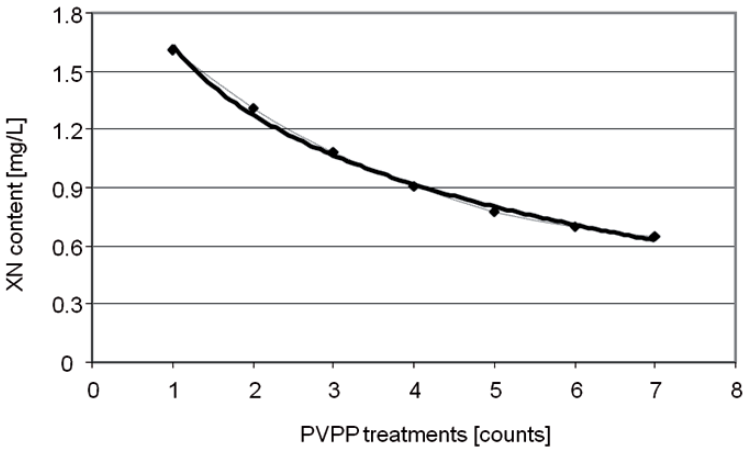

Fig. 11 Repeated treatment of XN enriched beer with PVPP

4 Conclusion

The presented results suggest that during the brewing process the point in time of XN dosage should be as late as possible at the end of boiling of pale and dark brews to optimise XN yield. Additionally, a fast cool down of wort should follow similar to 'XAN-technology' in dark brews too. This approach allows doubling or even tripling of XN contents in wort without additional colouring at equal XN dosage.

In final, dark beer, roasting substances support the XN stability during storage. The XN carrying substances arise from intensive roasting and belong to HMW substances. The type and strength of binding differs for roasting substances and equilibrium reactions affect the XN content in wort and beer. We presume that different roasting substances interact with functional groups of XN and therefore only parts of these functional groups are available for SPE or PVPP. Another conceivable reason for modified physical, chemical, and possibly biological properties may be an enclosure of XN similar to the effect observed for cyclodextrins (supra molecules). As consequence the dissolution rate, membrane permeability, and bioavailability of XN would change.

Beside a characterisation of XN carrying substances in more detail, a recommended daily intake for XN is in demand so that a reasonable XN content in beer can be put into practice. However, the results concerning the colour:XN ratio emphasis that in richly roasted malts more XN carrying substances occur. This ratio is low when (1) the appropriate brewing technology is applied or (2) a special XN rich RMB extrakt is used. It may further decrease by optimised roasting of the raw material. As consequence XN rich brews can be produced that show only very low additional colouring. This should match the preferences of customers for pale beers, so a larger market could be developed. Moreover, these procedures could enhance XN enrichment in beverages like non-alcoholic beers or wort based beverages and therefore offer oppertunities for product developer to create new XN containing drinks.

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